

## THE ACCUMULATION OF NICOTINE IN CONSTITUTIVE SALICYLIC ACID PRODUCING-TOBACCO AFTER WOUNDING AND TMV-INOCULATION OF LEAVES

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### ABSTRACT

Nugroho, L.H. 2001. The accumulation of nicotine in constitutive salicylic acid producing-tobacco after wounding and TMV-inoculation of leaves. *Biologi* 2(12): 724-733.

The accumulation of nicotine in the constitutive salicylic acid producing (CSA)- and non-transgenic tobacco (*Nicotiana tabacum* L. Samsun NN) plants after wounding and TMV-inoculation of leaves were detected using gas chromatography. The nicotine accumulation was suppressed in the TMV-inoculated leaves of CSA-tobacco, whereas wounding enhanced nicotine accumulation. Furthermore, in the time course study of the accumulation of nicotine and related alkaloids was found that no difference between CSA-tobacco and non-transgenic tobacco plants.

*Key words:* tobacco, nicotine, tobacco mosaic virus, leaf damage

### INTISARI

Nugroho, L.H. 2001. Akumulasi nikotin pada tembakau yang mengandung asam salisilat konstitutif-setelah perlakuan dilukai dan diinfeksi dengan TMV. *Biologi* 2(12): 724-733.

Akumulasi dari nikotin pada tembakau yang mengandung asam salisilat secara konstitutif (tembakau transgenik) dan tembakau yang bukan transgenik kultivar Samsun NN sesudah mendapat perlakuan dilukai dan diinfeksi dengan *Tobacco Mosaic Virus* (TMV) dideteksi dengan menggunakan kromatografi gas. Akumulasi nikotin mengalami penurunan setelah mendapat perlakuan diinfeksi dengan TMV pada tembakau transgenik, sedangkan perlakuan dilukai meningkatkan akumulasi nikotin baik pada tembakau transgenik maupun tembakau yang bukan transgenik. Pengamatan akumulasi nikotin dan alkaloid yang lain dari waktu ke waktu menunjukkan tidak adanya perbedaan antara tembakau transgenik dan tembakau yang bukan transgenik.

*Key words:* tembakau, nikotin, tobacco mosaic virus, daun luka

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### INTRODUCTION

Research was done at Division of Pharmacognosy, Leiden University, The Netherlands.

Different types of secondary metabolites found in leaves increase after leaf damages. Many of the damage-induced increases have proven to be related with a defense role of secondary metabolites (Baldwin, 1994). Alkaloid contents, mainly nicotine and nornicotine, in *Nicotiana sylvestris* increases to reach a maximum after 9 days and wane to control level at 14 days after leaf damage (Baldwin, 1989). Removal of flowering heads at the onset of flowering, a standard practice in cultivation of tobacco (*Nicotiana tabacum*), increases the alkaloid content of the leaves. The increase in leaf alkaloid content after removal of the flowering top is largely a result of increased nicotine synthesis in the roots (Mizusaki *et al.*, 1973). The alkaloids are then carried to the leaves by the xylem flow (Dowson, 1942).

Many experiments have proven that jasmonic acid (JA) is an essential component of the signal transduction cascade for the production of nicotine after leaf

damage (Baldwin, 1999). Moreover, Baldwin (1999) described that when rosette stage plants of *N. attenuata* are wounded or attacked by herbivores, the endogenous wound signal cascade producing JA is activated in wounded tissues, resulting in increased JA pools in the shoots. These, in turn, stimulate nicotine synthesis in the roots and increase nicotine pools throughout the whole plants.

The accumulation of JA and nicotine were inhibited by the application of salicylic acid (SA) directly to the wounded tissue. SA is known as an important signal compound activating plant defense responses against pathogen attack. However, the application of SA to unwounded tissue adjacent to the wound did not induce nicotine production (Baldwin, 1999). This suggested that the SA signal cascade may inhibit the JA signal cascade (Baldwin *et al.*, 1997 and Preston *et al.*, 1999).

The constitutive salicylic acid producing (CSA) tobacco (*Nicotiana tabacum* L. cv. Samsun NN) (Verberne *et al.*, 2000), were tested for the accumulation of alkaloids, mainly nicotine,

nornicotine, and anabasine. However, there was no difference in alkaloid accumulations between CSA-tobacco and non-transgenic tobacco (Nugroho *et al.*, 2002). In this article, we report the further study of the accumulation of nicotine in CSA-tobacco after wounding and TMV-inoculation of the leaves.

## MATERIALS AND METHOD

### Plant Materials

Thirty plants grown from T1-seeds of CSA tobacco (*Nicotiana tabacum* L. cv Samsun NN) line 15 (Verberne *et al.*, 2000) and 30 plants from the seeds of non-transgenic tobacco (*Nicotiana tabacum* L. cv Samsun NN) were grown in the greenhouse to the 4-5 leaf stage (about 2 months old). Three leaves in each plant of 10 CSA- and 10 non-transgenic tobacco plants were cut one-third using a pair of scissors. Three leaves in each plant of 10 CSA- and 10 non-CSA-tobacco plants were inoculated with TMV. The other 10 plants were used as controls. Five control plants were inoculated with PEN buffer. After 4 days treatment, the leaves were harvested for alkaloid extractions. The experiment was then repeated

for time course study by harvesting in 2, 4, 6, 8 and 10 days after treatments. Four plants for each treatment were used in the second experiment.

### TMV Inoculation

The TMV-inoculation was done according to the method of Enyedi *et al.*, (1992). Three fully expanded leaves were abraded with carborundum (400 grit) and inoculated with 40 µl TMV suspension by gently rubbing the upper leaf surface. Leaves were rinsed with deionized water following inoculation. The TMV suspension was prepared by diluted 3 µl TMV stock solution (60 µg/ml) in 10 ml PEN buffer (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1mM EDTA, 1 mM NaN<sub>3</sub>, pH 7). Lesions were evident within 2 days after inoculation.

### Alkaloid Extraction Procedure

The extraction of alkaloids was according to Pakhale and Maru (1998) with slight modifications. The leaves were grounded in a mortar with a pestle. The grinding was done in liquid nitrogen. Two grams of grounded material were extracted with 10 ml of 0.1% TFA

(Trifluoroacetic acid). After vigorously mixing with a vortex, the extract was sonicated for 30 minutes and then centrifuged at 4,000 g for 10 minutes to which isoquinoline had been added as an internal standard. After filtration, the filtrate was extracted twice with 15 ml dichloromethane. The organic layer was discarded and the pH of the aqueous phase was then adjusted to 11 with 1 M NaOH, and subsequently extracted 3 times with 15 ml each of dichloromethane-methanol (9:1, v/v). The aqueous layer was discarded. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and then concentrated using a rotavapor. The residue was redissolved in 500 µl MeOH for the GC-analysis.

### Gas Chromatography

The samples were analysed with an Agilent 6890 series gas chromatograph equipped with an Agilent 7683 series automatic injector and a nitrogen-phosphorous (NPD) flame ionization detector. The alkaloids were analyzed by using 30:1 split-injection on 30 m X 0.32 mm HP1 Chrompack column (temperature program 100 - 250°C at 5°C/min, constant flow-velocity, 25 cm/sec

of nitrogen). The injection-port temperature was 240°C, the detector temperature was 325°C and 1 µl samples were injected. The NPD-response curves were obtained by analysis of a series of standard solutions. (S)-(-)-Nicotine (98% purity), nornicotine (97% purity) and anabasine (85% purity) purchased from Sigma-Aldrich Chemie GmbH, Germany were used as standard compounds and isoquinoline (97% purity) which was also purchased from Sigma-Aldrich Chemie GmbH, Germany was used as internal standard. The signals were integrated using an Agilent 6890 integrator.

### Analysis of SA and SA-glucoside

The extractions of SA and SA-glucoside (SA-G) were done using the methods of Verberne (2000). Salicylic acid was quantified using HPLC. The HPLC system consisted of a 2150 HPLC pump from LKB, a Rheodyne 7010 injector with a 100 µl loop and a Shimadzu RF-10Axi spectrofluorometric detector. All analyses were performed at room temperature on a Phenomenex column, type LUNA 3µ C18 (2) 150 x 4.60 mm 3 µm, with a SecurityGuard from Phenomenex. The eluent con

sisted of 0.2 M sodium acetate, pH 5.5 and 10% methanol with a flow rate of 0.80 ml/min. SA was detected in an emission and excitation wavelength of 407 nm and 305 nm respectively.

## RESULTS AND DISCUSSION

The result that there was no effect of constitutive salicylic acid production on the accumulation of nicotine and related alkaloids in tobacco (*N. tabacum* L. Samsun NN) was obtained (Nugroho *et al.*, 2002). Here, we did a further experiment on the effect of wounding and TMV-inoculation on CSA-tobacco and non-transgenic tobacco leaves. After 4 days treatment, the damaged leaves, TMV-inoculated leaves, PEN-buffer inoculated leaves, and control leaves of CSA-tobacco and non-transgenic tobacco were harvested for the analysis of alkaloids. The results show that the nicotine accumulation in wounded leaves of CSA-tobacco and non-transgenic tobacco plants increased up till six-fold compared to control leaves of CSA-tobacco and non-transgenic tobacco plants (Figure 1). However, there was no difference in nicotine accumulation between

CSA-tobacco and non-transgenic tobacco plants after leaf wounding. It might be that the accumulation of SA ( $1.16 \pm 0.28 \mu\text{mol}$ ) in the leaves of CSA-tobacco was not sufficient to inhibit the production of nicotine. Suggesting that SA was unable to inhibit the signal cascade of JA, known to play an important role in the signal transduction cascade for the production of nicotine in *Nicotiana* (Baldwin, 1999). Furthermore, the nicotine accumulation in the leaves of CSA-tobacco and of non-transgenic tobacco increased two-fold after the inoculation with PEN-buffer (Figure 1). The increase in nicotine accumulation in the leaves of CSA-tobacco and non-transgenic tobacco may be due to the carborundum wounding, as after PEN buffer was applied, the leaves were abraded with carborundum. However, the accumulation of nicotine after inoculation with PEN-buffer was not as high as in leaves damaged by cutting. This result is in agreement with Baldwin (1989) who stated that there was a strong positive relationship between the kind of leaf-wounding and the amount of nicotine accumulation.

When CSA-tobacco and non-transgenic tobacco plants were inoculated with TMV, nicotine accumulation was suppressed in CSA-tobacco (Figure 1). Preston *et al.*, (1999) suggested that inhibition of the JA signal cascade may only occur when SA is applied at high concentration. It may be that the SA level in CSA-tobacco after TMV inoculation ( $3.95 \pm 0.39 \mu\text{mol}$ ) was sufficient to suppress the signal cascade of JA, resulting in lower nicotine accumulation.

The CSA-plants produce SA constitutively in the leaves at a level comparable to a TMV-infected non-transgenic plant. Obviously this level in CSA plants does not effect the biosynthesis and accumulation in healthy plants. Only after infection with TMV the CSA plants show lower nicotine accumulation. This fits with the previous observations that the CSA plants have a normal phenotypes but reach differently (more resistant) after viral or fungal infections. Targeted metabolic profiling thus is an important quantitative and qualitative tool for characterizing phenotypes.

All experiment conditions, except the control plants were wounded with PEN buffer, were

then repeated for a time course study. The leaves of CSA-tobacco and non-transgenic tobacco plants were harvested at day 2, 4, 6, 8 and 10 after the treatments. The results show that there was no difference in the trend of nicotine and related alkaloids accumulation between CSA-tobacco and non-transgenic tobacco in the time course study. The accumulation of nicotine and related alkaloids reached a maximum at day 8 after the leaf damage (Figure 2a, b). This result was in accordance with those of Baldwin (1989). Moreover, the trend of nicotine and related alkaloids accumulation in CSA-tobacco and non-transgenic tobacco after TMV-inoculation were quite constant from day 2 to day 10. However, the data of day 10 from non-transgenic tobacco could not be collected due to the dryness of the leaf (Figure 3a, b). The constant alkaloids accumulation from day 2 to day 10 might due to the antagonism between SA and JA signal cascade (Creelman and Mullet, 1997).

From all of the results, it can be concluded that TMV infection suppressed the accumulation of nicotine in CSA-tobacco. There was no difference of nicotine and related alkaloids accumulations

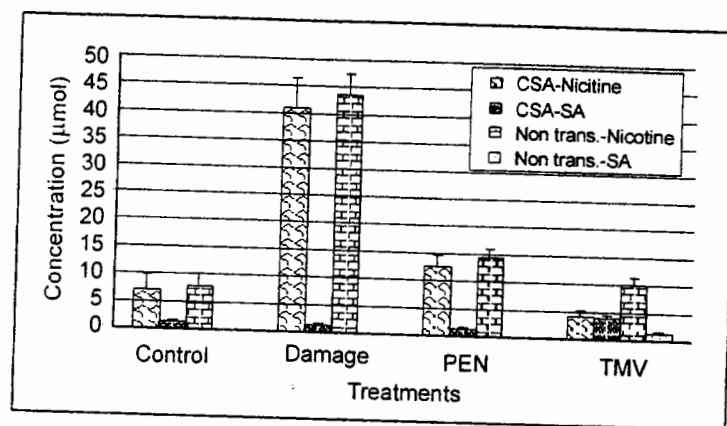


Figure 1. The accumulation of nicotine and salicylic acid in the leaves of CSA-tobacco and non-transgenic tobacco (*N.tabacum* L.Samsun NN) after 4 days treatments. Damage: the leaves were damaged by cutting (n=10 plants), PEN: the leaves were damaged with PEN-buffer (n=5 plants), TMV: the leaves were inoculated with TMV (n=10 plants), Control: leaves without any treatments (n=5 plants). The nicotine data were calculated based on internal standard (isoquinoline) in 2 gram fresh weigh leaves and the salicylic acid data were calculated from the sum of free SA and SA after acid hydrolysis in 1 gram of fresh weigh leaves.

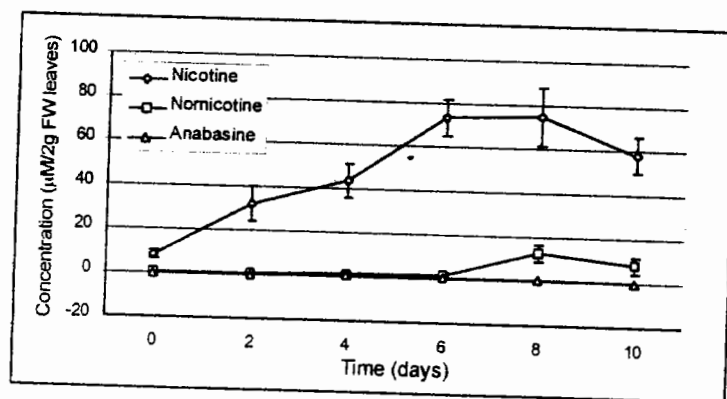


Figure 2a. The accumulation of nicotine and related alkaloids in non-transgenic tobacco (*N. tabacum* L.Samsun NN). The leaves were harvested at different day after leaf damaged (n = 4 plants). Data was calculated based on internal standard (isoquinoline).

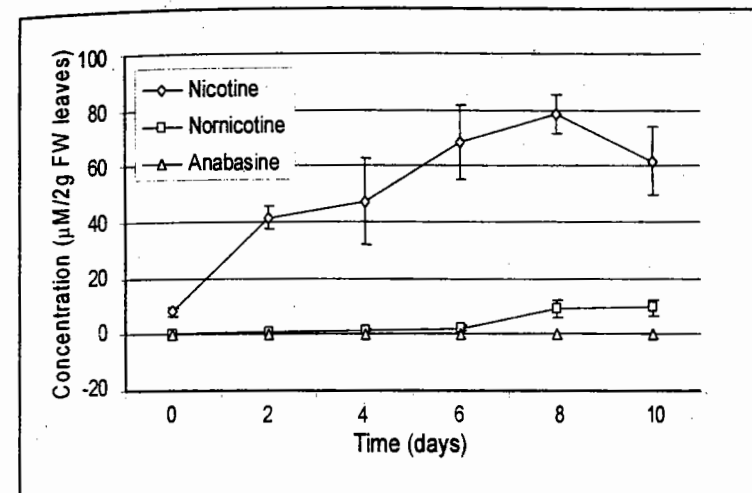


Figure 2b. The accumulation of nicotine and related alkaloids in CSA-tobacco (*N.tabacum* L.Samsun NN). The leaves were harvested at different day after leaf damaged (n = 4 plants). Data were calculated based on internal standard (isoquinoline).

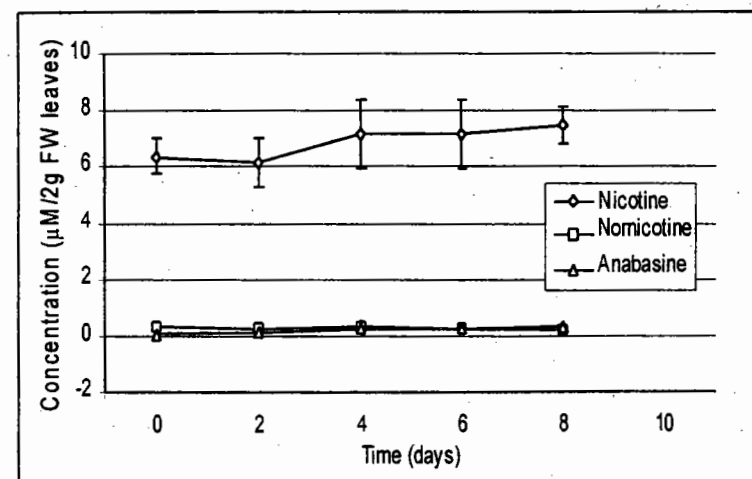


Figure 3a. The accumulation of nicotine and related alkaloids in non-transgenic tobacco (*N. tabacum* L.Samsun NN). The leaves were harvested at different day after TMV inoculation (n = 4 plants). Data were calculated based on internal standard (isoquinoline).

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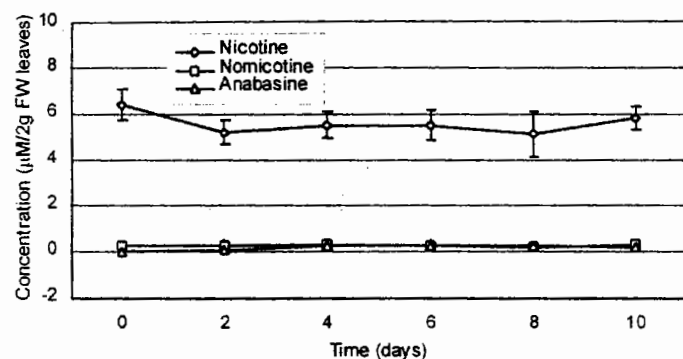


Figure 3b. The accumulation of nicotine and related alkaloids in CSA-tobacco (*N. tabacum* L. Samsun NN). The leaves were harvested at different day after TMV inoculation (n = 4 plants). Data were calculated based on internal standard (isoquinoline).

in CSA and non-transgenic tobaccoduring the time-course study after various treatments.

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